PRODUCTION OF POLY(3-HYDROXYBUTYRATE) FROM PINEAPPLE WASTE

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ABSTRACT

P(3HB) is the potential replacement of synthetic plastics due to its biodegradability and sustainability. Pineapple waste is one of the example of food waste that capable to produce P(3HB) as they contains sucrose, glucose, fructose and other nutrients that essential for production of bio plastics. Thus, the aim of this research is to employ the sugar present in pineapple waste as the carbon source for the bacteria producing biopolymer. The objectives are as follow; to determine the sugar content in the pineapple waste juice, to study the effect percent volume of juice from pineapple waste on production of P(3HB), to study the effect of pH on P(3HB) production. The method involve in this study are cultivation of bacteria, preparation of pineapple waste, preparation of inoculum, P(3HB) biosynthesis in shake flask, cell dry weight analysis and analysis of P(3HB) using HPLC. The results showed that glucose content in the mixture of skins and pulps is the highest with total of 28.80 g/L compared to fructose and sucrose. Fructose content come second with 21.25 g/L while sucrose has the lowest content in the PWJ with 20.28 g/L. The pineapple waste juice (PWJ) is then used as medium for the production of P(3HB). The fermentation medium with 50% and 100% volume of PWJ has the highest concentration of P(3HB) of 2.01 g/L (30^{th} hour) and 1.49 g/L (44^{th} hour) respectively. As the total dry mass production, the medium with 75% of PWJ has the highest concentration of P(3HB) with 14.42 g/L. The total concentration of P(3HB) for 50% and 100% PWJ are 10.52 g/L and 4.42 g/L respectively. The total average concentration of P(3HB) produced in the medium at pH 7 is 8.97 g/L. The highest concentration was found to be at the 44^{th} hours with 2.15 g/L. As conclusion, the medium that consists of 75% PWJ and 25% ultra-pure water with the mixture of nutrient rich element at pH 7 is the most suitable medium to produce P(3HB).

ABSTRAK

P(3HB) berpotensi sebagai pengganti plastik sintetik kerana biodegredasi dan kelestariannya. Sisa nanas merupakan salah satu contoh bahan buangan makanan yang mampu untuk menghasilkan P(3HB) kerana mereka mengandungi sukrosa, glukosa, fruktosa dan nutrien lain yang penting untuk pengeluaran plastik bio. Oleh itu, tujuan kajian ini adalah untuk menggunakan gula dalam sisa nanas sebagai sumber karbon bagi bakteria menghasilkan biopolimer. Objektif adalah seperti berikut; untuk menentukan kandungan gula dalam jus sisa nanas, untuk mengkaji peratus jumlah kesan jus daripada sisa nanas pada pengeluaran P(3HB), untuk mengkaji kesan pH ke atas penghasilan P(3HB). Kaedah ini melibatkan dalam kajian ini ialah penanaman bakteria, penyediaan bahan buangan nanas, penyediaan inokulum, P(3HB) biosintesis kelalang, sel kering analisis berat dan analisis P(3HB) menggunakan HPLC. Hasil kajian menunjukkan bahawa kandungan glukosa dalam campuran kulit dan pulpa adalah tertinggi dengan sejumlah 28.80 g/L berbanding fruktosa dan sukrosa. kandungan Fruktosa datang kedua dengan 21.25 g/L manakala sukrosa mempunyai kandungan yang paling rendah dalam jus sisa nanas (JSN) dengan 20.28 g/L. JSN telah digunakan sebagai medium untuk pengeluaran P(3HB). Medium penapaian dengan 50% dan 100% jumlah JSN mempunyai kepekatan tertinggi P(3HB) daripada 2.01 g/L (jam 30) dan 1.49 g/L (jam 44), masing-masing. Oleh kerana jumlah pengeluaran besar-besaran yang kering, sederhana dengan 75% daripada JSN mempunyai kepekatan tertinggi P(3HB) dengan 14.42 g / L. Jumlah kepekatan P(3HB) untuk 50% dan 100% masing-masing JSN 10.52 g / L dan 4.42 g / L. Jumlah purata kepekatan P(3HB) yang dihasilkan dalam jangka sederhana pada pH 7 adalah 8.97 g / L. Tertinggi didapati pada waktu ke-44 dengan 2.15 g/L. Kepekatan tertinggi didapati pada waktu ke-44 dengan 2.15 g/L. Kesimpulannya, medium yang terdiri daripada 75% JSN dan 25% air ultra tulen dengan campuran unsur kaya nutrien pada pH 7 adalah medium yang paling sesuai untuk menghasilkan P(3HB).

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LIST OF ABBREVIATIONS

$CuCl_2.2H_2O$	Copper(II) chloride
$CuSO_4.5H_2O$	Copper (II) Sulfate Pentahydrate
$CrCl_3.6H_2O$	Chromium(III) chloride
H_2SO_4	Sulfuric Acid
KH_2PO_4	Monopotassium Phosphate
K_2HPO_4	Dipotassium Phosphate
HPLC	High Performance Liquid Chromatography
LCA	Life Cycle Assessment
$MgSO_4.7H_2O$	Magnesium Sulfate
$MnCl_2.4H_2O$	Manganese(II) Chloride Tetrahydrate
$Na_2MoO_4.2H_2O$	Sodium molybdate
NaOH	Sodium Hydroxide
$NiCl_2.6H_2O$	Nickel(II) chloride
PHA	Polyhydroxyalkanoates
P(3HB)	Poly(3-Hydroxybutyrate)
PP	Poly Propylene
PWJ	Pineapple Waste Juice
$(NH_4)_2SO_4$	Ammonium sulfate
$ZnSO_4.7H_2O$	Zinc Sulfate Heptahydrate

1 INTRODUCTION

1.1 Motivation and statement of problem

Plastics or known as polypropylene are the important ingredients that increase the quality of our life. Its production is increased since 1940s and able to replaces wood, metals, glass, mud and other materials (Hamieh *et al.*, 2013). Due to its low cost, durability, stability, good mechanical and thermal properties make it used worldwide (Amara, 2008).

The extensive usage of materials from plastics causes a world problem due to their degradability (Muller et al., 2001). Some synthetic plastics that widely used nowadays take hundred years to degrade. The traditional plastics produced from petrochemical sources that can be depleted and takes over millions of years to be renewed. The synthetic plastic contributes to the environmental problems as they go to the water streams. Many campaigns were conducted to create the awareness to reduce the usage of synthetic plastic. Somehow, the campaign still not able to reduce the usage of plastic since it is useful and needs for people. A huge increment of the synthetic plastics accounted from year 2006 to 2009 (Snell and Peoples, 2009).

In order to reduce the usage of these synthetic plastics, there are rapidly increment in the development and production of environment-conserved biodegradable plastics (Chee et al, 2010). Current issues about the global environment and solid waste management already catches intention to develop biodegradable plastics that should be produced in a large scale for worldwide use (Anderson and Dawes, 1990). Besides the concern about the environment issue on the degradability of synthetic plastics, the exhaustion of fossil fuels also led to the study on the production of biodegradable plastics from renewable sources (Aarthi and Ramana, 2011).

Some options for the replacement of synthetic plastics are bio-based materials such as polynucleotides, polyamides, polysaccharides, polyoxoesters, polythioesters, polyanhydrides, polyisoprenoids and polyphenols (Steinbuche, 2001). Amongst these, polyhydroxyalkanoate (PHA) from the group of polyoxoesters has got the attention from the industries due to their thermoplastics properties and biodegradability of biopolymers (Albuquerque, 2007).

Patel *et al.* (2003) stated that, in order to compare the environmental performance of biopolymers with petrochemical plastics, a standardized method to quantify environmental impacts which is the tools of life cycle assessment (LCA) can be applied. According to several studies that have been conducted recently, it shows that the production of PHAs was more beneficial in a full cradle-to-gate LCA compared to the production of petrochemical analogues poly(ethylene) and PP (Akiyama *et al.,* 2003; Harding *et al*., 2007; Pietrini *et al.,* 2007).

PHAs are thermoplastic polymer that synthesized by various types of bacteria as energy storage materials and intracellular compounds as the shortage of nutritional elements and excess of carbon source (Baei, 2009). Some bacteria have ability to produce PHA up to 90% (w/w) of dry cells during the lacking of essential nutrients such as magnesium, nitrogen and phosphorus (Madison and Huisman, 1999).

Even though there are more than 250 natural PHA-producers, only a few of them have been employed for the biosynthesis of PHA including *Alcaligenes latus, B. megaterium, C. necator* and *P. oleovorans* that have capabilities to utilize various carbon sources to produce PHA (Chee *et al.,* 2010). Among these bacteria, *C. necator* is the best bacteria known as PHA producing microorganisms. There are large proportion of the tested bacteria was able to produce PHA but the amounts of PHA produce are low for the industrial applicants (UGUR *et al.,* 2002).

Although the biodegradable plastics already publish in the market for a few years; due to high production cost, the uses of PHA are limited even though they have been recognized as good replacement of synthetic plastics (Steinbuchel, 1996). The high production cost of PHA has been a primary weakness to their replacement of petrochemical plastics (Byrom, 1987; Choi and Lee, 1997). The production cost of synthetic plastics that made from petroleum such as polyethylene and polypropylene are significantly lower than PHA (Lee, 1996). Almost half of the total production cost goes to raw materials in which the carbon source took 70-80% of the total expense (Choi and Sang, 1997). So it is preferable to develop a production process by a waste carbon source to reduce the total production cost.

The performance of the bacterial fermentation and the cost of the final product are critically depending on the selection of the carbon sources (Chee *et al.,* 2010). The basic criterion in choosing the carbon sources are the availability, low cost, renewable and carbon sources that can support the microbial growth and produce PHA effectively.

The waste materials that are discharged from agriculture and food processing industries are the potential renewable carbon sources for the production of PHA.

Several organic compound as carbon source to produce PHA has been identified such as soya waste (Yu *et al.*, 1999), beet molasses (Omar *et al.*, 2001), and palm oil (Marsudi, 2008). As the complex starch substrates were used directly without involvement of any hydrolysis step, the production of the P(3HB) from inexpensive carbon sources in the form of starch is advantageous (Aarthi and Ramana, 2011). Pineapple waste can potentially be used as carbon source for organic acid fermentation based on their physio-chemical properties (Busairi, 2009).

As mentioned by Moon and Woodroof (1986), in pineapple canning process, the solid waste was estimated about 40-50% from the fresh fruit are skins and cores. Furthermore, if these wastes are discharged to the environment, they may cause an environment problem.

1.2 Objectives

The aim of this research is to employ the sugar present in pineapple waste as the carbon source for the bacteria producing biopolymer. The objectives are as follow:

i. To determine the sugar content in the pineapple waste juice.

ii. To study the effect percent volume of juice from pineapple waste on production of P(3HB).

iii. To study the effect of pH on P(3HB) production.

1.3 Scope of this research

The fermentation carried in this study will be done in 500 mL shake flask. Prior to that, the sugar content in the pineapple waste juice will be analysed using HPLC. In order to study the effect of sugars concentration on P(3HB) production, various concentration in the range of 10-60% will be examine in shake flasks experiment.

1.4 Organisation of this thesis

The structure of the reminder of the thesis is outlined as follow:

Chapter 2 provides a brief description about the background of the study. This chapter also provide the information about what is P(3HB), the advantages and disadvantages of P(3HB), the applications of P(3HB), the microorganisms used and the selected carbon source; pineapples.

Chapter 3 provides a brief description of the methodology to achieve the three objectives in the study. This chapter is divided into experimental method, research materials and analytical method. Experimental method consists of 6 steps which are cultivation of bacteria, preparation of pineapple waste, determination of sugar content in pineapple juice, preparation of inoculum 1, preparation of inoculum 2 and P(3HB) biosynthesis in shake flask. The analytical methods are cell dry weight analysis and analysis of P(3HB) using HPLC. A summary of the previous experimental work on production of P(3HB) is also presented.

Chapter 4 is devoted to a summary of the results achieved from the experimental procedure. This chapter also consists of the discussion and comparison from the experimental results with previous study done by others.

Chapter 5 provides the conclusion achieve from the study. This chapter also provides the recommendation for future study.

2 LITERATURE REVIEW

2.1 Overview

This paper presents the brief description about the background of the study. This chapter also provide the information about what is P(3HB), the advantages and disadvantages of P(3HB), the applications of P(3HB), the microorganisms used and the selected carbon source; pineapples..

2.2 What is P(3HB)?

Poly (3-hydroxybutyrate) [P(3HB)] is a linear polyester of D(-)-3-hydroxybutyrate acid was first figured by a French scientist, Lemoigne in 1925 and it is the most common polyhydroxyalkanoate (PHA) (Doi, 1990). P(3HB) is a biopolymer that found in prokaryotes, in which it serves as a reserve of carbon and energy (Slepecky and Law, 1961). The polymer, which serves as a reserve of carbon and energy, is now known to be a general class of compounds referred to as polyhydroxyalkanoates and possessing the general formula as in Figure 2.1.

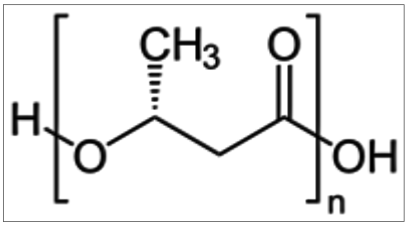

Figure 2.1: Molecular structure of P(3HB)

PHB is a highly crystalline thermoplastic polymer with a high melting temperature within the rage of 170-180°C and has a glass transition temperature between 0-5°C.

P(3HB) compound which is known as rather brittle, highly crystalline and high melting aliphatic polyester with similar properties to many industrial polyolefins especially poly (propylene) (PP) is the best and most common PHA (Atlic *et al.*, 2011). However, unlike polyolefins, the PHAs can be synthesized from the renewable feedstock include agricultural wastes by industrial (white) biotechnology method. At the

end of their service life as converted to plastic items, they can either taken as a source for the production of valuable chiral building blocks (3-hydrocy acids) by chemical or enzymatic treatments or can undergo fast and complete biodegradation by microorganisms in solid and aqueous media (Anderson and Dawes 1990; Braunegg *et al.* 1998; Lee and Lee 2003; Reddy *et al.* 2003; Khanna and Srivastava 2005; Ren *et al.* 2005).

The recognition of P(3HB) as the bacterial storage polymer that have almost similar function to starch and glycogen was accepted at 1973 (Dawes and Senior, 1973). According to Dawes and Senior (1973), P(3HB) is accumulated in the intracellular granules by various types of Gram-positive and Gram-negative organisms under limitation of nutrients besides carbon source. The intracellular degradation of P(3HB) is occurred in the absence of carbon and energy sources (Macrae and Wilkinson, 1959).

In order to improve the poor low-impact strength of P(3HB), the incorporation of hydroxyvalerate monomers into the polymer which produce polyhydroxybutyrate-co-valerate (PHBV); commercially marketed under the trade name Biopol. These PHBV also, like P(3HB) completely degrades into carbon dioxide and water under aerobic conditions.

Bsides *C. necator*, a number of microorganisms able to acts as P(3HB) bacteria producers such as *Pseudomonas guezennei*, *Escherichia coli (E. coli),* and *Bacillus megaterium* (Christelle *et al.*, 2008; Wang and Lee, 1997; Macrae and Wilkinson, 1958). Chee *et al*. (2010) reported that some of the bacteria that are capable to utilize many types of carbon sources including plant oils or wastes to produces PHA are *Alcaligenes latus, B. megaterium, C necator and P. oleovorans.*

2.3 Advantages of P(3HB)

The most known characteristics of P(3HB) is its biodegradability in various environment. Many microorganisms in environments that excrete PHA depolymerizes have ability to hydrolyze PHA into water soluble oligomers and monomers that can be used as nutrients by living cells (Kim, 2000). Features of PHA such as its biodegradable, water insoluble, non-toxic, biocompatible piezoelectonic, thermoplastic

and elastomeric make them suitable to be use in the packaging industry and suitable as hydrocarbon-based plastics (Halami, 2008; Anderson and Dawes, 1990).

Besides that, P(3HB) can be used as biodegradable thermoplastic material for management strategies of waste in the medical stream (Steinbuchel, 1995; Mona et al., 2001). P(3HB) is an ideal carbon reserve material since it exists in the cell in a highly reduced state as a virtually insoluble polymer exerting negligible osmotic pressure (Dawes, 1988). P(3HB) is very suitable to be used as food packaging material as it is insoluble in water, resistant to ultraviolet radiation and it is impermeable to oxygen (Aarthi and Ramana, 2011). Furthermore, P(3HB) possesses a better physical properties than polypropylene for food packaging applications and it is completely nontoxic (Hankermeyer and Tjeerdema, 1999).

2.4 Disadvantages of P(3HB)

Pure PHB is a high crystalline thermoplastic, rather brittle and not sufficiently flexible for some purpose (Dawes, 1973). P(3HB) has a high fragility, showing 3-5% tensile elongation at break, and a low thermal stability above its melting point, with marked degradation from 200°C.

Humidity, temperature and the dwell time in the machine during the processing of PHB could cause the polymer to degrade in the final products such as films, coatings or fibres (Endres and Siebert-Raths, 2011).

2.5 Applications of P(3HB)

The potential application of P(3HB) to replace the synthetic plastics is high due to its biocompatibility, biodegradability and almost negligible toxicity to the cells. P(3HB) has been produced for polymer films, non-woven materials, sutures and pharmaceutical products that being used in surgery, tissue engineering, transplantology and pharmacology.

The P(3HB) has attracted the medical attention as it is being used as capsule for controlled drug release, filaments as surgical sutures and the cotton wool products as swabs (Dawes, 1973). P(3HB) are suitable to be used as biodegradable material for implantation inside human body (Ghosh, 2011).

2.6 Microorganisms

The microorganisms choose for this study is *Cupriavidus necator*. Chee (2010) mentioned that *C. necator* has been widely studied and used as bacterium for PHA production. *C. necator* or kown as *Alcaligenes eutrophus* is bacteria that has potential of producing SCL-PHA and identified able to produce PHA polymers consist of 3HB, 3HV and 4HB monomers (Doi, 1990; Kunioka *et al.*, 1989; Saito *et al.*, 1996).

C. necator have ability to produce up to 66% (w/w) of P(3HB) with glucose as the carbon source (De Rooy *et al.,* 2007). Doi *et al*. (1987) stated that *C. necator* as the main P(3HB) producing microorganism.

2.7 Pineapple

Agricultural wastes such as pineapple waste has been identified have potential to be used as raw materials for conversion into useful added-products (Jamal et al., 2009). Cultivated pineapple (*Ananas comosus* (L.) Merril or *Ananas comosus var comosus*) belong to *Bromeliaceae* family and originated from South Africa. Roslina (2008) reported that the pineapple waste contains high content of carbohydrate that can be used as carbon source for the production of organic acid. Busairi (2009) reported that the liquid pineapple waste has potential to be used as carbon source for organic acid fermentation as they contain sucrose, glucose, fructose and other nutrients.

In tropical region such as Malaysia, Thailand and Indonesia, there are huge pineapples canning industries that are produce large quantity of solid and liquid waste. In the canneries, nearly 75% of the fruit in the form of peeled skin, crown, core, pulp and others is discharged as wastage and create problems of disposal and pollution (Busairi, 2010).

Apart of their pollution and hazard treats, food processing waste such as pineapple waste might have a potential for recycling as a raw material or for conversion into useful product of higher value added products, raw material for other industries, as well as for use as food or feed after biological treatment (Kroyer, 1991).

The pineapple skin waste was figured out to have 10% amount of reducing and 13% amount of non-reducing sugars that encourage the growth of microorganisms (Dhanasekaran, 2011). Based on the study by Hemalatha and Anbuselvi (2013), the

pineapple skin waste has higher amount of sugar (9.75%), non-reducing sugar (8.8%) and protein content (10mg) than the juice, which is suitable for the growth of microorganism.

3 MATERIALS AND METHODS

3.1 Overview

This chapter consists of the methodology to achieve the three objectives in the study. This chapter is divided into experimental method, research materials and analytical method. Experimental method consists of 6 steps which are cultivation of bacteria, preparation of pineapple waste, determination of sugar content in pineapple juice, preparation of inoculum 1, preparation of inoculum 2 and P(3HB) biosynthesis in shake flask. The analytical methods are cell dry weight analysis and analysis of P(3HB) using HPLC. In order to determine the sugar content in pineapple waste by comparing with the stock solution of glucose, sucrose and fructose; HPLC is used. Based on the sugar content in the pineapple waste, the dilution is made to prepare the samples with various concentration of pineapple waste based on the sugar content. The synthesis of P(3HB) is carried in the shake flask for 48 hours. The samples are collect every 6 hours for cell dry weight analysis. The next analysis to determine the production of P(3HB) is carried out by HPLC by comparing the sample with standard P(3HB) prepared.

3.2 Experimental methods

The experimental method involve in 6 stages:

1. Cultivation of bacteria
2. Preparation of pineapple waste
3. Determination of Sugar Content in Pineapple Juice
4. Preparation of inoculum 1
5. Preparation of inoculum 2
6. P(3HB) biosynthesis in shake flask

3.2.1 Cultivation of bacteria

The bacteria choose for this study is *Cupriavidus necator*. C. necator (CCU 52338) has ability to accumulate about 60% of P(3HB) with glucose as the carbon

source (De Rooy *et al.*, 2007). The bacteria are transfer from the culture stock to petri plate by streaking method. The plate was incubated at 30°C for 24 hours (Baei *et al.*, 2009).

Table 3.1: Nutrient agar medium element

Chemicals	Amount (g)
Peptone	5
Glucose	10
Yeast extract	3
Agar	15
Nutrient broth	8
Pure water	1000 ml

(Source: Zahari *et al.*, 2012)

3.2.2 Preparation of Pineapple waste

The pineapple is get from any fruit seller nearby. The pineapple is peeled and the waste (skins/peels) is used as the main materials in this study. The pineapple skins (100g) are washed and cut into smaller pieces before blending with 100 mL distilled water (Jamal *et al.*, 2009, Zakaria and Nazeri, 2012).

After that, the juice is centrifuged at 15000 rpm for 15 minutes at 4°C (refrigerated centrifuge). The supernatant is filtered using mixed cellulose ester membrane filter with pore size, 3 to 5µm (Zahari *et al.*, 2012). The juice is then sterilized using an autoclave.

Figure 3.1: Pineapple peels and pulps

3.2.3 Determination of Sugar Content in Pineapple Juice

The concentration of sugar contain in the pineapple waste juice is determine by High Performance Liquid Chromatography (HPLC). The HPLC was operated at following conditions (Pongjanta *et al.*, 2011):

Table 3.2: Operating condition of HPLC

Mobile phase	Acetonitrile: Deionized water (3:1)
Detector	Reflective index detector (RID)
Flow rate	1 ml/min
Column temperature	30°C
Detector temperature	35°C

The standard solutions at 10, 20, 30, 40 and 50 g/L of glucose, sucrose and fructose are prepared for calibration (Ersoy *et al.*, 2007). Acetonitrile was added to each standard solution to obtain a similar composition as in the mobile phase (3:1). The prepared standard solution is then filtrate with 0.45 µm membrane filter.

The diluted samples and the sugar standard were filtered using Nylon membrane 0.45 µm and injected directly to the reverse phase chromatography column (Ersoy *et al.*, 2007).

3.2.4 Preparation of inoculum 1

The 24 hours incubated bacteria from petri plate is transferred into 5 ml of sterile nutrient rich medium in test tube. Total of six test tubes are used. This step was carried out in laminar air flow with aseptic technique applied. The test tubes are then incubated in incubator shaker for 24 hours. The temperature is set to 30°C at 200 rpm.

Table 3.3: Nutrient rich medium element

Chemicals	Amount (g)
Peptone	1.0
Glucose	2.0
Yeast extract	0.6
Nutrient broth	1.6
Pure water	200 ml

(Source: Zahari *et al.*, 2012)

3.2.5 Preparation of inoculum 2

24 hours of incubated culture from test tube is transferred into 15 ml of sterile nutrient rich medium in 100 mL flask. Each test tube is transferred into a flask to have a total of 20 ml. The pH value is adjusted to 7.0 using 5 M NaOH and 5 M of H_2SO_4. The flask is then incubated for another 24 hours at 30°C and 200 rpm. The inoculum is used as 10% of the fermentation medium. (Zahari *et al.*, 2012).

3.2.6 P(3HB) biosynthesis in shake flask

In order to study the effect of percent volume of juice from pineapple waste on production of P(3HB), the different amount of pineapple waste juice are added for the fermentation. Based on the total sugar of the pineapple waste juice, the concentration of pineapple juice involved are 50%, 75% and 100%.

In flask of 50% concentration, 100 ml of juice is mixed with 80 ml of pure water. In the flask of 75% concentration, 150 ml of juice is mixed with 30 ml of pure water. Meanwhile, for the flask of 100% concentration, the mineral salt medium is diluted directly with 200 ml of juice. Each concentration is duplicated to get an average. The MSM stock solution is also prepared. All solution prepared is autoclave at 121°C for 15 minutes. The pH of the solution is adjusted to 7 before autoclave.

Table 3.4 Mineral salt medium (MSM) element

Chemicals	Amount (g)
KH_2PO_4	6.7
$(NH_4)_2SO_4$	1.0
K_2HPO_4	1.5
$MgSO_4.7H_2O$	0.2
Pure water	100 ml

(Source: Zahari *et al.*, 2012)

After autoclave, 20 ml of sterile MSM stock solution is added to the flask of 50% and 75% concentration respectively. This step is carried out in laminar air flow with sterile condition. Then, the 20 ml of pre-grown cells is added to each flask. Incubate the culture at 30°C and 200 rpm for 48 hours before harvest the sample.

In order to study the effect of pH on the P(3HB) production, the solutions prepared are represented the acid (pH 6), base (pH 8) and neutral (pH 7). As in the second objective; effect of percent volume, the solutions prepared are in neutral condition (pH 7).

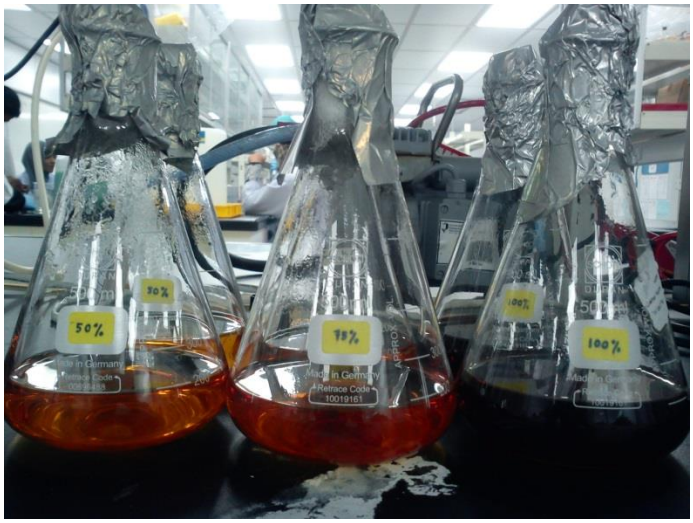

Figure 3.2: The pineapple juice with different concentration after autoclave

3.3 Research Materials

The materials used in this research were peptone, glucose, yeast extract, agar, nutrient broth, KH_2PO_4, K_2HPO_4, $(NH_4)_2SO_4$, $MgSO_4.7H_2O$, $NiCl_2.6H_2O$, $CuCl_2.2H_2O$, $ZnSO_4.7H_2O$, $MnCl_2.4H_2O$, $Na_2MoO_4.2H_2O$, $CuSO_4.5H_2O$, $CrCl_3.6H_2O$, and pineapple waste juice.

3.4 Analytical Method

1. Cell dry weight analysis
2. Analysis of P(3HB)

3.4.1 Cell dry weight analysis

10 mL of the sample is taken every 6 hours. The microfuge tube is weighed before use. 1 mL of the sample is filled inside the tube and then centrifuge at 10 000 rpm for 5 minutes. The supernatant is removed and cell is washed using deionized water. The microfuge tube is dried in oven at 60°C for 48 hours before it is weighed again. (Rosslan, 2013).

Cell dry weight,

$$= \frac{\text{X (g)} - \text{Y (g)}}{\text{Volume (L)}}$$

X=Weight of microfuge tube containing dry pellet (after 48 hour)

Y=Weight of empty microfuge tube (before dry)

3.4.2 Analysis of P(3HB)

The dried microfuge tube is filled with 1 mL of concentrated H_2SO_4 and heat in oven at 90°C for 2 hours. Microfuge tube is vortex to mix the solution. The solution is

mixed with 9 mL of ultra-pure water in a test tube and vortex again. 1 mL of the solution is filled in vial after filtration.

To prepare the mobile phase, 0.426 mL of H_2SO_4 is mixed with 2L of ultra-pure water. The solution is filtered using vacuum pump filter and use ultrasonic to remove the bubbles.

To prepare the standard P(3HB),0.1g of P(3HB) standard is put into microfuge tube and 1 mL of H_2SO_4 is add. Then, heat it at 90°C for 2 hours. Transferred the solution into 1L volumetric flask before adding 1L of ultra-pure water. 1 mL of the standard solution is filtered using 0.45µm filter and place into vial. The sample then tested using high performance liquid chromatography (HPLC) (Rosslan, 2013).